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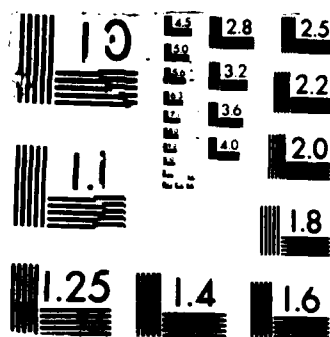
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Effects of Immunomodulatory Drugs on T Lymphocyte Activation and Function

Annual Report

Constantine D. Tscukas, Ph.D.

June 15, 1987

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19. lymphocyte surface proteins upon activation. The surface markers we tested were the CD3 molecular complex, the CD2 protein (sheep red cell receptor), the helper and cytotoxic cell markers CD4 and CD8, respectively, and the CD25 receptor (IL-2 receptor). In these experiments lymphocytes were activated with lectin Concanavalin A in the presence or absence of the various drugs. The expression of surface markers was then assessed by indirect surface immunofluorescence and cytofluorographic analysis. Comparisons were made among groups that received drugs and the no drug controls. The results of these experiments indicated that three of the drugs had selective effects. CL 246 inhibited the expression of CD25 while it had no effect on the other markers. OK 432 caused decrease in CD2 and increase in CD4 and CD25 expression. Finally, WY 18251 increased CD4 expression. The above results reveal some interesting effects of these drugs in in vitro immunologic functions.

SUMMARY

During the past year, the following drugs were received and tested for their immunomodulatory actions: CL246 738, AbuMDP, Xerosin II, OK 432, CL259 763, FK 565, AVS 2141, AVS 2142, AVS 2143, and AVS 2144. We tested the effects of these drugs on the proliferative activities of human T lymphocytes. To this end, lymphocytes were activated with the T cell specific mitogenic monoclonal antibody OKT3 which reacts with the CD3 molecular complex on human T cells and causes them to proliferate and secrete lymphokines. Lymphocytes isolated from the blood of healthy volunteers were incubated with monoclonal antibody OKT3 in the presence or absence of various concentrations of the different drugs. These studies revealed that three of the drugs, CL246, Xerosin, and AVS 2143 when used at specific concentrations increased the proliferation of lymphocytes. Five of the drugs, FK 565, OK 432, WY 18251, AVS 2141, and AVS 2142 had the reverse effect, namely they reduced proliferation. Finally, three of the drugs, CL259, AbuMDP, and AVS 2144 had no effect. We also tested the effects of the same drugs on the expression of selected lymphocyte surface proteins upon activation. The surface markers, we tested were the CD3 molecular complex, the CD2 protein (sheep red cell receptor), the helper and cytotoxic cell markers CD4 and CD8, respectively, and the CD25 receptor (IL-2 receptor). In these experiments lymphocytes were activated with the lectin Concanavalin A in the presence or absence of the various drugs. The expression of surface markers was then assessed by indirect surface immunofluorescence and cytofluorographic analysis. Comparisons were made among groups that received drugs and the no drug controls. The results of these experiments indicated that three of the drugs had selective effects. CL 246 inhibited the expression of CD25 while it had no effect on the other markers. OK 432 caused decrease in CD2 and increase in CD4 and CD25 expression. Finally, WY 18251 increased CD4 expression. The above results reveal some interesting effects of these drugs in in vitro immunologic functions.

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FOREWARD

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

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BODY OF REPORT

In the present progress report, we summarize our data of testing the effects of various drugs, provided by the Department of Antiviral Studies, on human T lymphocyte activation.

During this phase of the study, we have assessed the effects of the drugs on the proliferation of T cells which is induced by monoclonal antibodies to the CD3-antigen receptor complex. The CD3-antigen receptor molecular complex is found on the surfaces of all mature human T lymphocytes (1). It is composed of a group of proteins two of which are a disulfide-linked heterodimer while the rest (at least three distinct proteins) are non-covalently associated with each other (2). The heterodimeric component serves the function of antigen recognition and binding (antigen receptor) and the non-covalently associated members (CD3) are believed to play the role of intracellular signal transducers (3-5). Monoclonal antibodies reactive with proteins of the complex induce proliferation of T cell, production of Interleukin 2, and expression of Interleukin 2 receptors (6-8).

In addition, we have assessed the effects of the drugs on the expression of T lymphocyte surface proteins. T lymphocytes express on their surfaces a variety of proteins which primarily serve the function of receptors and as such play an important role in cellular activation (9). An example of these T cell surface proteins is the CD3-antigen receptor complex mentioned above. We have also assessed the effects of the drugs on the expression of certain other surface proteins which are thought to play important roles in T cell activation. The surface proteins we tested are the receptors for Interleukin 2 (CD25) because of their importance in binding and mediating the biologic growth effects of Interleukin 2 (10), the receptor for sheep red blood cells (CD2) due to its involvement in T cell activation via an alternative pathway, and cytotoxic cell associated molecules CD4 and CD8, respectively (12). The latter two proteins are known to be involved in the recognition of gene products coded by the major histocompatibility complex; class I for CD8 and class II for CD4 (13).

In order to test the effects of the drugs on proliferation, peripheral blood lymphocytes were cultured with either an optimal (1/25,000) or suboptimal (1/200,000) dilution of the anti-CD3 antibody OKT3 in the presence of various concentrations of drugs. The cells were incubated for three days at 37°C and then proliferation was measured by the incorporation of ³[H]-thymidine into cellular DNA. The results of these experiments are displayed in Figures 1 to 10 of Appendix I and Table 1 of Appendix II. They are also summarized in Table 4 of Appendix II. CL 259, AbuMDP and AVS 2144 do not have any effect on T cell proliferation (Figures 1, 3, and 9 in Appendix I). AVS 2141 and AVS 2142 had a negative regulatory effect (Figures 6 and 7 in Appendix I). However, this effect was quite subtle. AVS 2143 displayed a biphasic effect by augmenting proliferation at low concentrations of drug (Figure 8 in Appendix I). Similarly, WY 18251 had a biphasic effect with highest suppressive effect at the lowest concentrations tested (Table 1 in Appendix II). CL 246 augmented proliferation at low concentrations only when suboptimal amounts of OKT3 were used for activating the T cells (Figure 2 in Appendix I). When this drug was added in the cultures at a concentration of

0.004 $\mu\text{g/ml}$ an approximately 2.25-fold increase in proliferation was seen. FK 565 and OK 432 (Figures 4 and 5 in Appendix I) displayed a negative effect on the proliferation while Xerosin (Figure 10B, Appendix I) mediated augmentation of the proliferative response. The latter was a dose dependent phenomenon and it was observed only with suboptimal amounts of OKT3 in the culture. In experiment 1 (Figure 10B, Appendix I) Xerosin at a concentration of 3 $\mu\text{g/ml}$ induced a 7-fold increase in proliferation. Similarly, in experiment 2 (same Figure) Xerosin at 1 $\mu\text{g/ml}$ induced 4-fold increase in proliferation.

In additional studies, the augmenting effects of Xerosin was further investigated. In the experiment displayed in Figure 11 of Appendix I, we asked the question whether the augmentation was due to the drug alone or there was a synergistic effect between the action of the drug and the OKT3 antibody in culture. The results displayed (Figure 11, Appendix I) indicate that the effect is clearly synergistic.

In other experiments, we tested the possibility whether CL246.738 can augment the synergistic effects of Xerosin on lymphocyte proliferation. Thus, we activated lymphocytes with antibody OKT3 in the presence of Xerosin with or without CL246. The addition of CL246 had no additional augmenting effect over the one observed with Xerosin (Table 2 in Appendix II).

Activation of lymphocytes with monoclonal antibody OKT3 involves the participation of both T cells and monocytes (14). We have found, however, that the requirement for monocytes can be substituted by the additional of IL-2 (15). Thus, the augmenting effects of Xerosin could be mediated via monocytes, T cells or perhaps both. We tested the possibility that the effects of Xerosin are mediated exclusively on T cells. To this end, we isolated T cells by rosetting with sheep red blood cells and further purified them by subsequent treatment with carbonyl iron and adherence (15,16). The purified T cells were then activated by addition of antibody OKT3 and IL-2 in the presence or absence of Xerosin. The antibody, IL-2 or Xerosin alone induced no significant proliferation (Table 3) in Appendix II). However, the combination of OKT3 and IL-2 together produced a strong proliferative signal (Table 3 in Appendix II). This proliferation was augmented when Xerosin was added in the cultures (Table 3 in Appendix II). The augmentation, however, was not of the same magnitude as the one with whole blood lymphocytes. This data suggest that Xerosin may have effects on both T cells and monocytes.

In other studies, we tested the effects of the same drugs on selected T lymphocyte surface markers upon activation. The markers we tested and the monoclonal antibodies utilized were the following: CD3 with antibody OKT3, CD2 with antibody OKT11, CD8 with antibody OKT8, CD4 with antibody OKT4 and CD25 with antibody to IL-2 receptor. The experimental protocol was as follows: human peripheral blood lymphocytes isolated by Ficoll-Hypaque density centrifugation were activated *in vitro* with concanavalin A (1 $\mu\text{g/ml}$) for 2 days at 37°C with or without addition of drug (1 $\mu\text{g/ml}$). These conditions were optimal as determined by preliminary experiments. Following culturing, the cells were collected and washed with α -methyl mannoside, the target sugar residue for concanavalin A, in order to remove any lectin from the surface of the lymphocytes. Subsequently, aliquots of cells were placed in microtiter tray wells and incubated with 1 $\mu\text{g/ml}$ of each monoclonal

antibody indicated above. A second fluorescein-conjugated goat anti-mouse antibody was added, and after additional incubation and washing the cells were analyzed in the cytofluorograph.

The results are shown in Figures 1 to 4 of Appendix III and are also summarized in Table 4 of Appendix II. They are displayed as histograms of fluorescence (horizontal axis) versus cell number (vertical axis). The open areas represent the negative controls and the filled areas represent the positively fluorescing cells. Each figure represents a separate experiment, each with its own 'no drug' control and 'drug' experimental groups. Sections A and B of each figure represent parts of the same experiment thus, the same control is utilized in analysis of data.

In the following discussion, all references to figures of data are those in Appendix III. Of eleven drugs tested, CL246 had a significant effect on the expression of IL-2 receptors, CD25 (compare panel B6 to panel A6 in Figure 1A). This effect seemed to be specific since drug treatment did not affect the other T lymphocyte surface markers (Figure 1A). OK432 depressed the expression of CD2 (compare panel D-T11 in Figure 2B to panel A-T11 in Figure 2A). In contrast, the same drug augmented the expression of CD4 (compare panel D-T4 of Figure 2B to panel A-T4 of Figure 2A). Similarly, the same drug augmented the expression of IL-2 receptors compare panel D-IL-2 of Figure 2B to panel A-IL-2 of Figure 2A. Finally, WY 18251 caused the elevation of CD4 expression (compare panels T4 between 'drug' and 'no drug' groups of Figure 3A).

It should be emphasized that the absolute percentages of positive cells, staining with a particular antibody, varied from experiment to experiment. This is a common observation and it probably represents experimental and donor to donor variability. It is, therefore, important that in analyzing the data, we compare each 'drug' group to its appropriate 'no drug' control in the particular experiment.

In conclusion, the up to date results of the *in vitro* screening of potentially immunomodulatory drugs is summarized in Table 4 (Appendix II). The most interesting results are displayed by CL 246 and Xerosin. Both seem to mediate a significant and reproducible augmentation of T cell proliferation. It is of interest, however, that CL 246 has the reverse effect on the expression of IL-2 receptors. This difference in effect is not clearly understood at the present time. However, it seems to represent a selective effect of this drug in T lymphocyte activation. Xerosin does not affect the expression of IL-2 receptors. It would be of interest to investigate the effects of these two drugs on production of IL-2 itself as well as other parameters of lymphocyte function. These experiments are part of the overall proposal and are planned for the future. Finally, the recommendation is made that drugs without any effects on the parameters tested so far be eliminated from further testing.

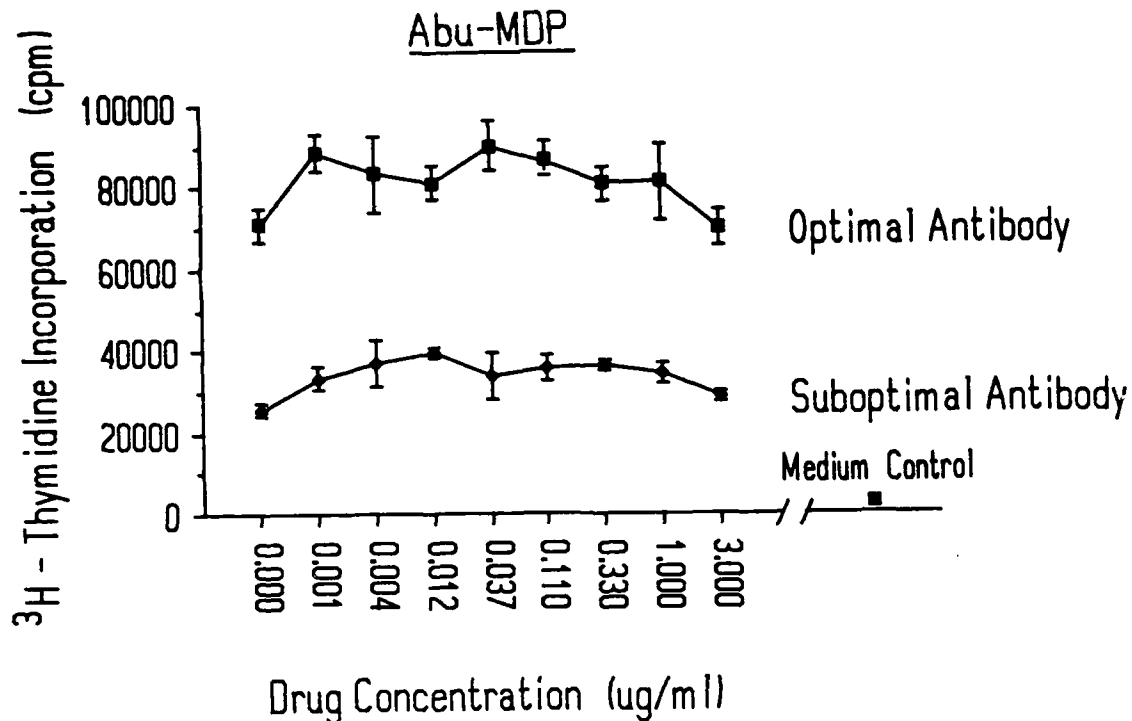
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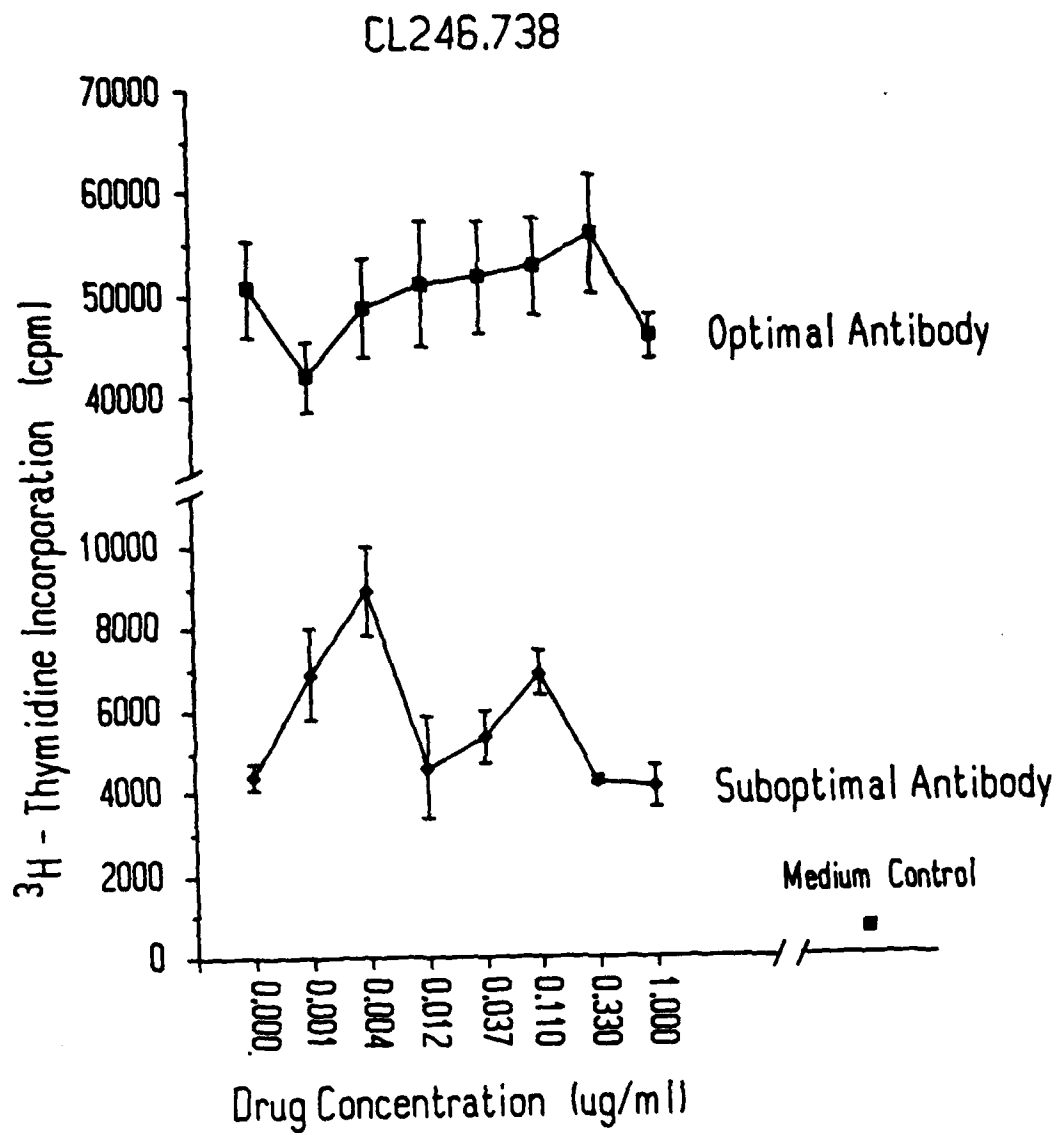
APPENDIX I

FIGURE 1



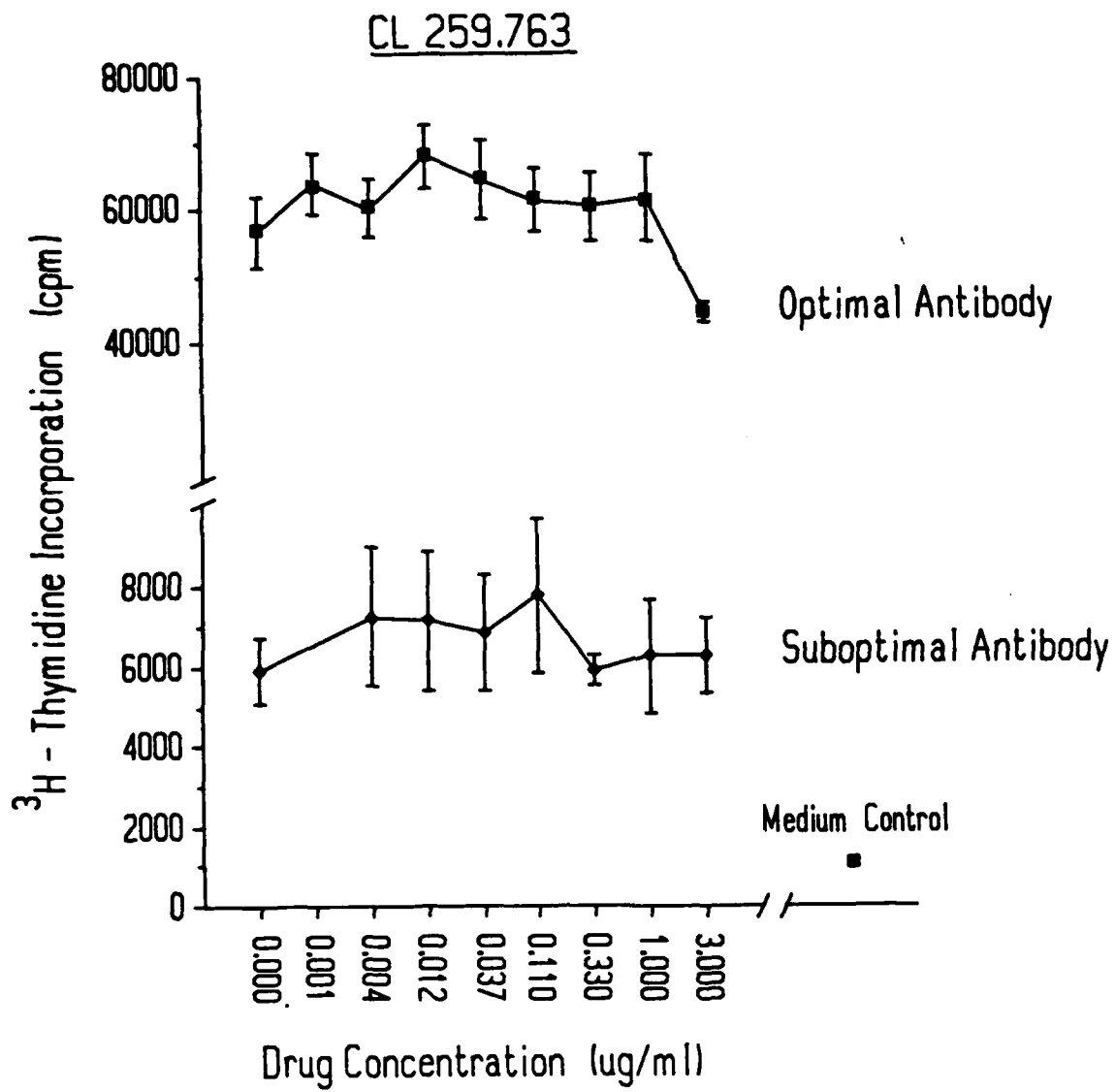
Human peripheral blood lymphocytes were incubated at 2×10^5 cells per microtiter tray well with monoclonal antibody OKT3 and various concentrations of the indicated drug. Five hours before the end of a 3-day incubation period, ^3H -thymidine ($1 \mu\text{C}/\text{ml}$) was added and incorporation of radioisotope in cellular DNA was assessed. The OKT3 antibody was in the form of culture medium supernatant obtained from a specific hybridoma. "Optimal antibody" was 1/25,000 dilution of OKT3 hybridoma supernatant. "Suboptimal antibody" was 1/200,000 dilution of supernatant. The negative control included media without antibody.

FIGURE 2



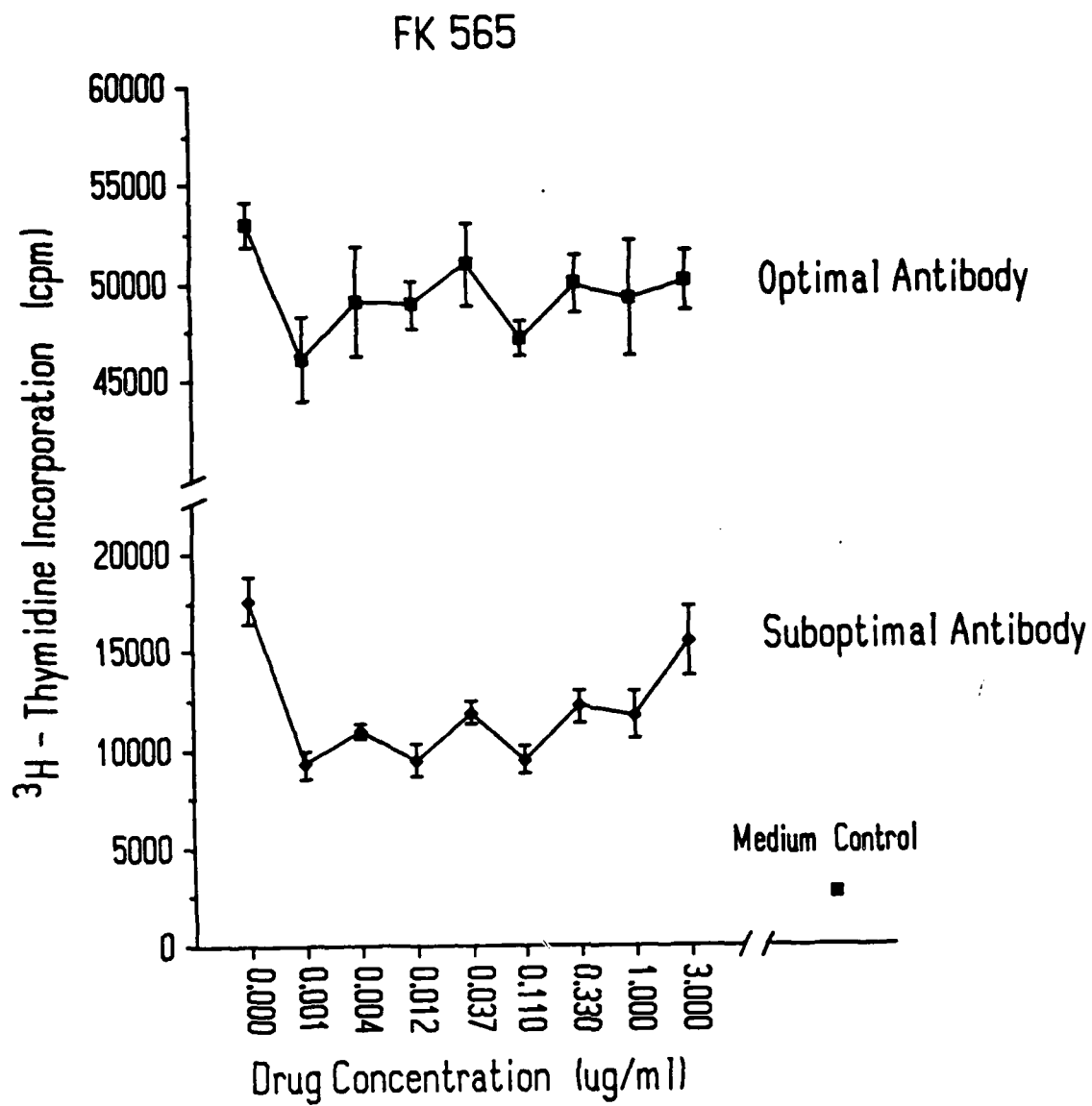
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FIGURE 3



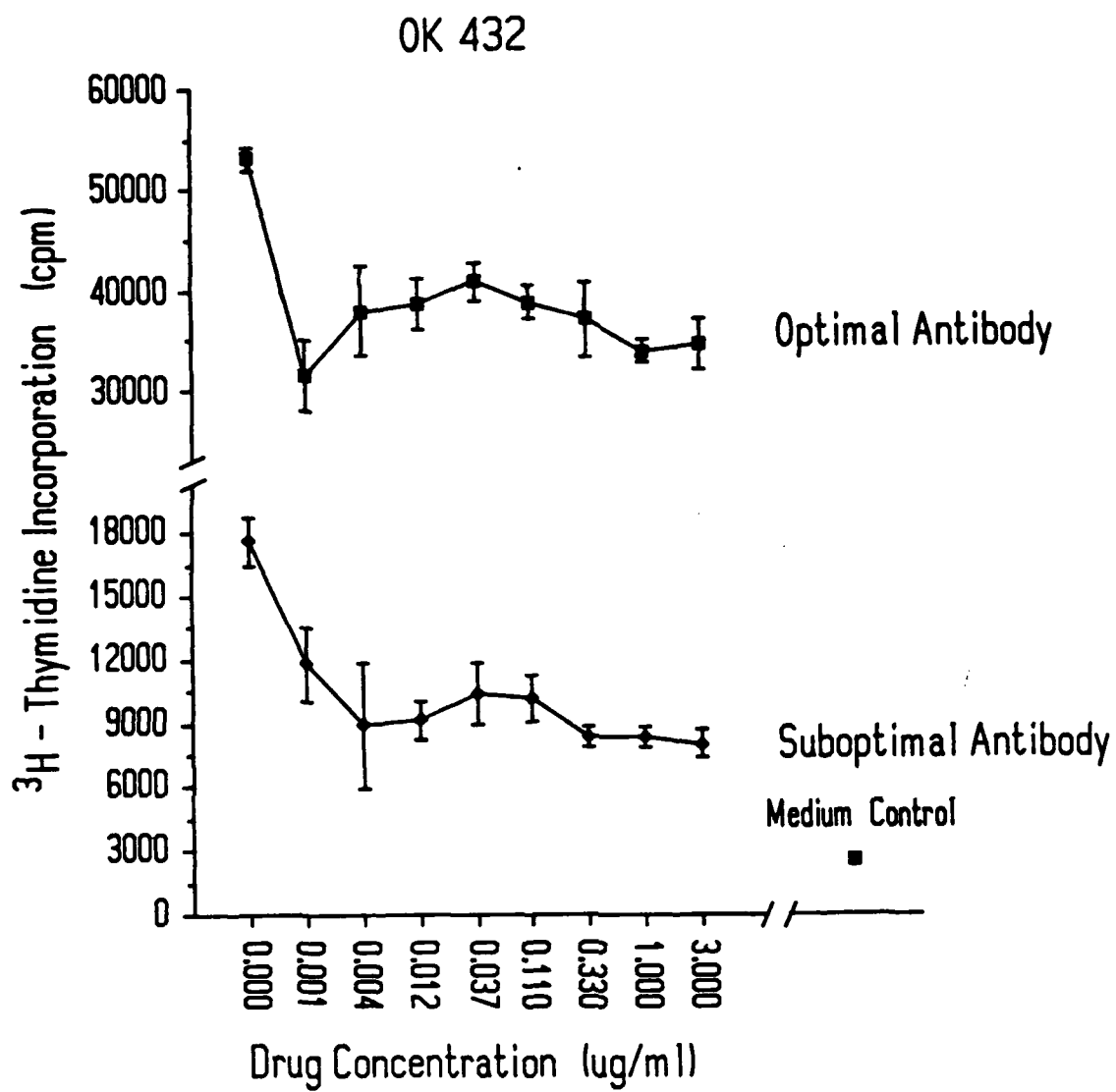
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FIGURE 4



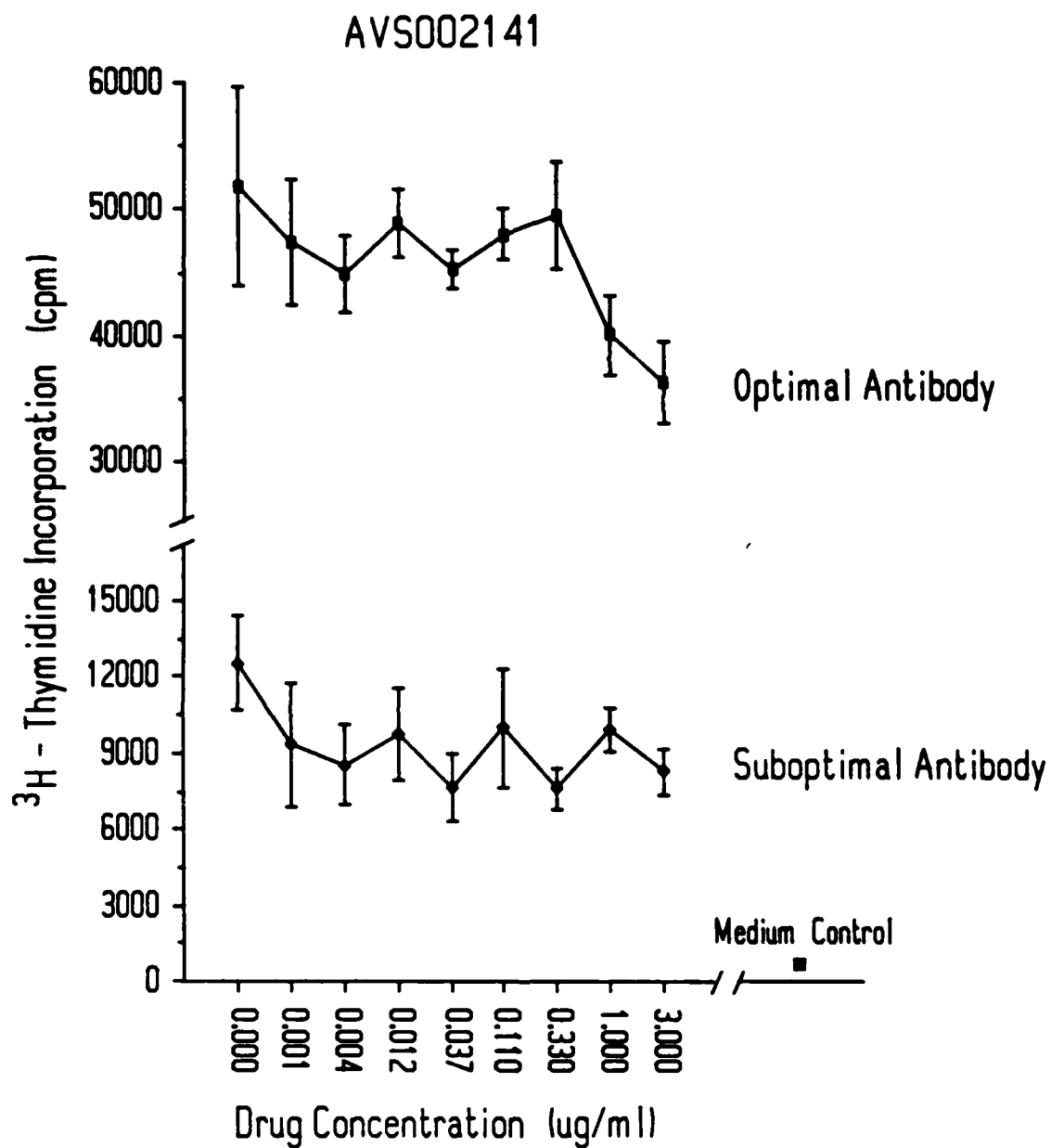
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FIGURE 5



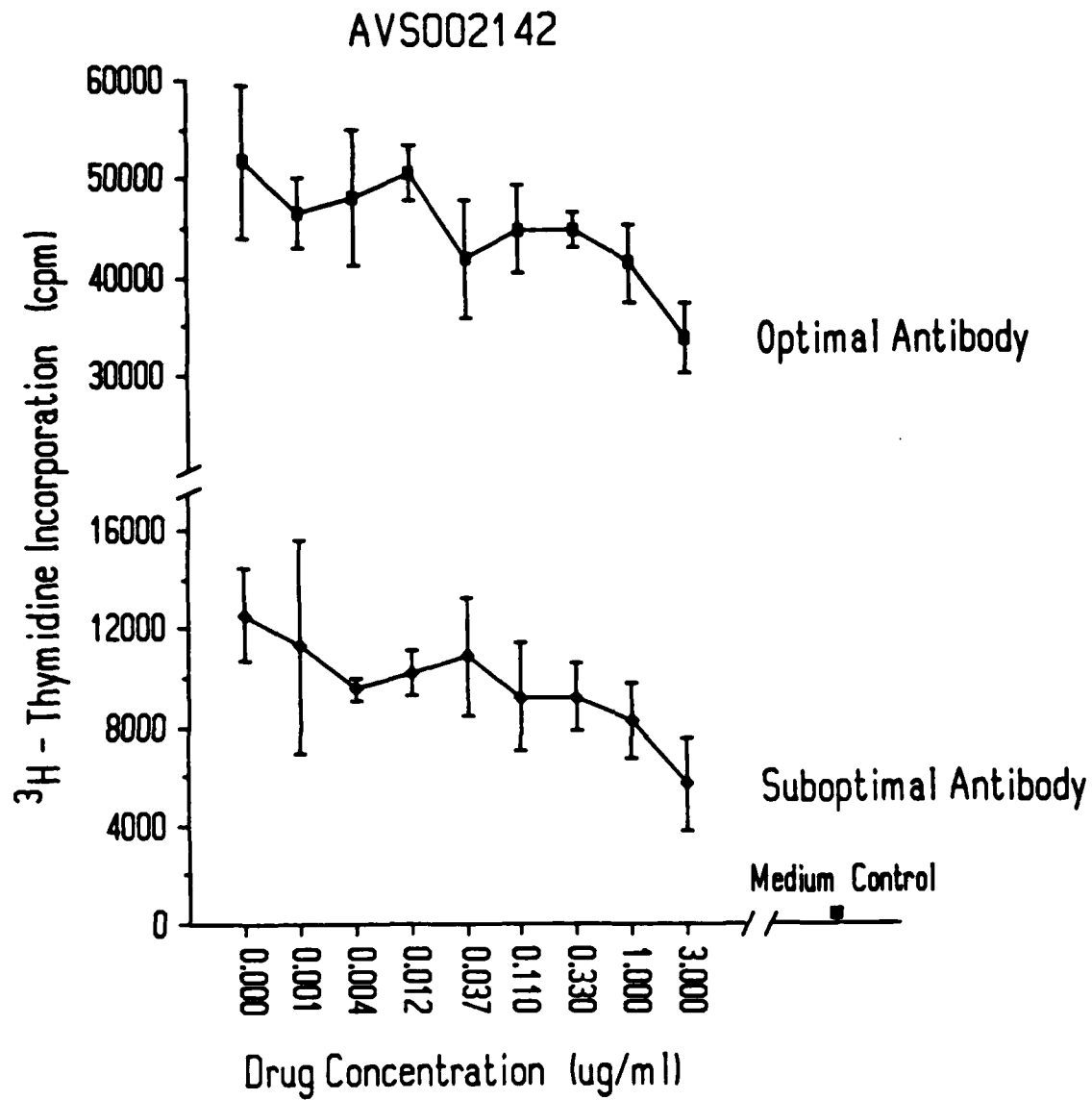
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FIGURE 6



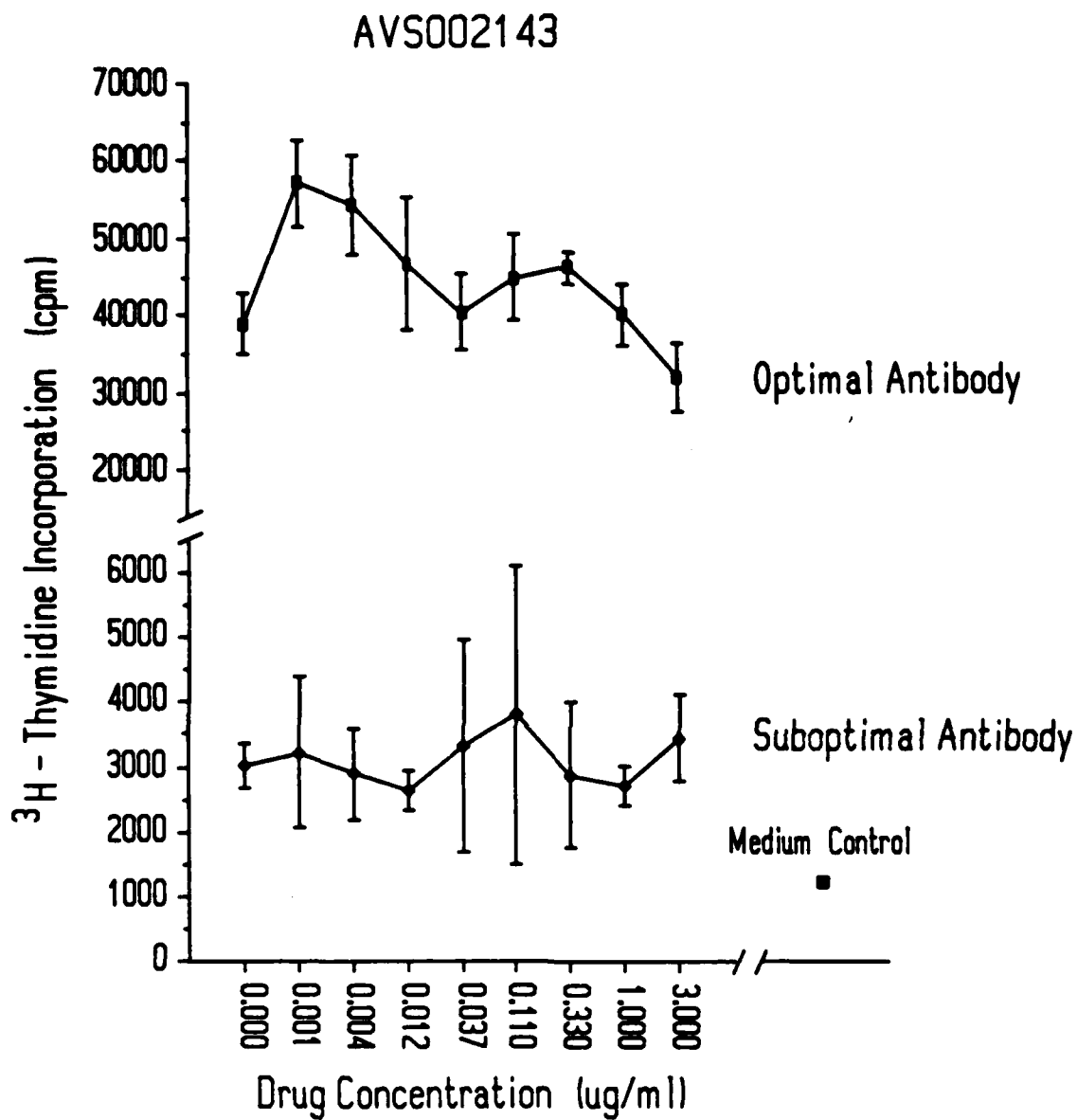
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FIGURE 7



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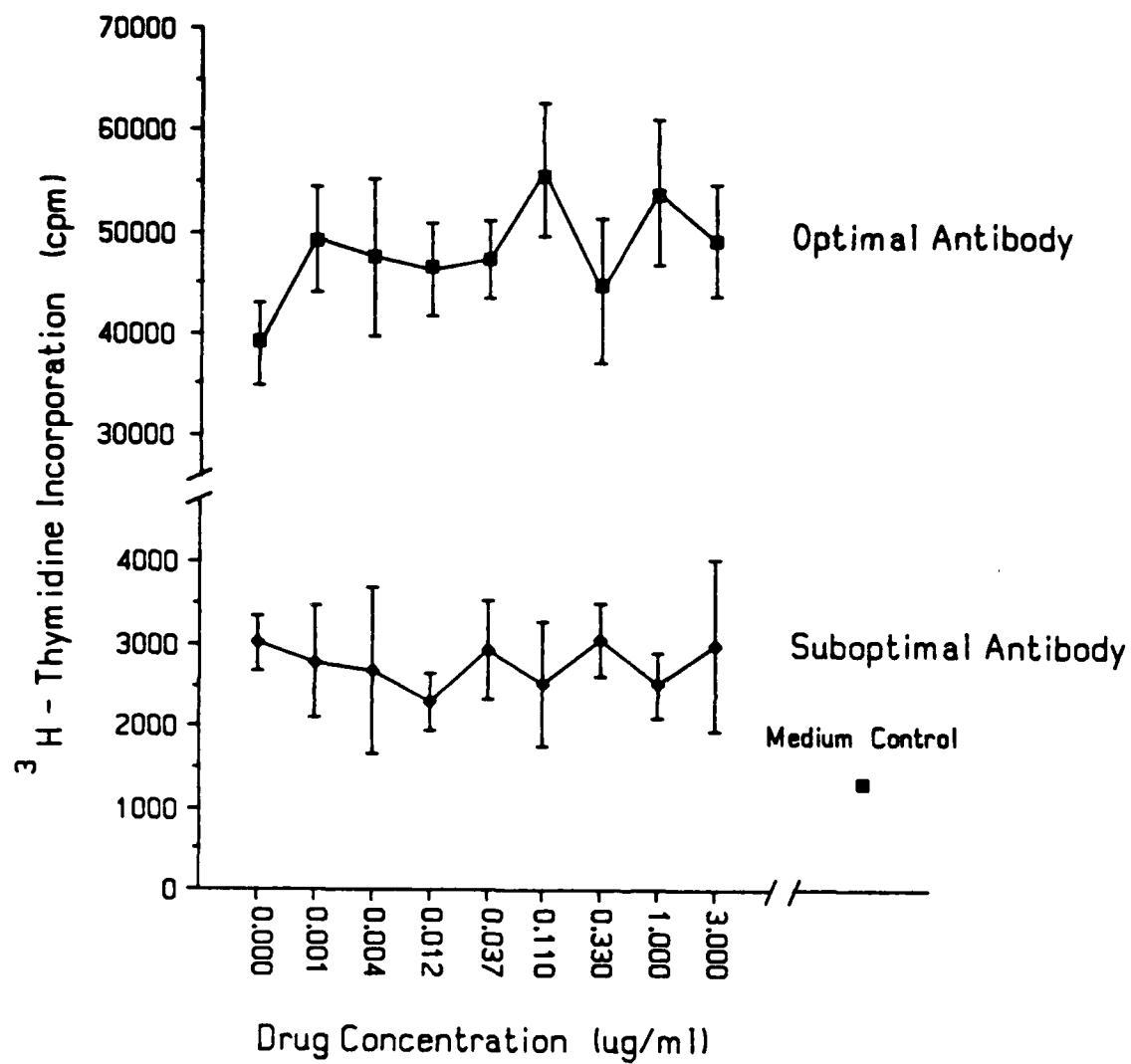
FIGURE 8



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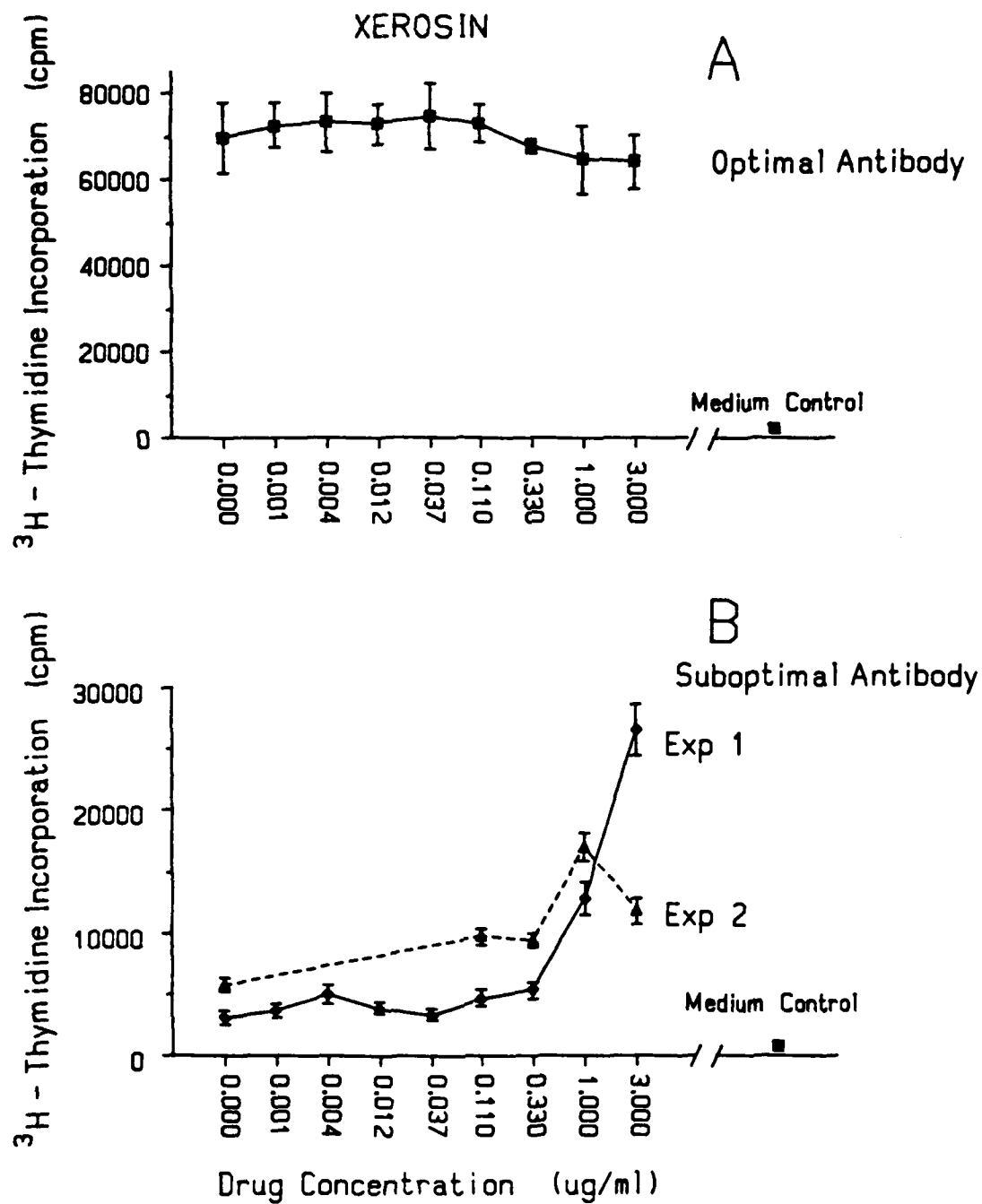
FIGURE 9

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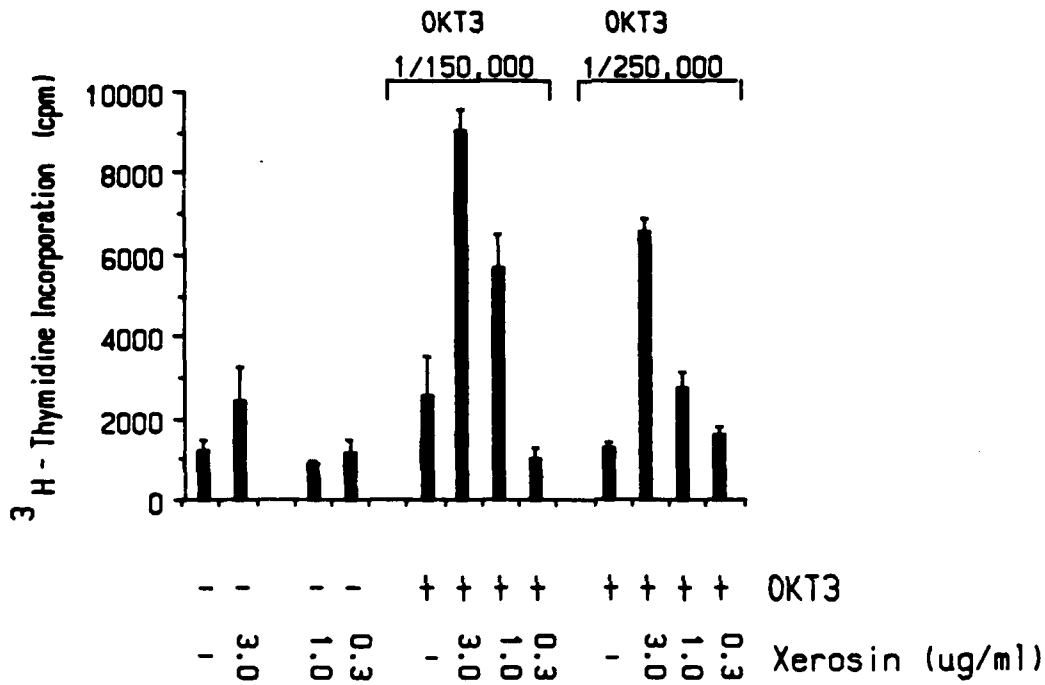
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FIGURE 10



SEE LEGEND OF FIGURE 1

FIGURE 11



Peripheral blood lymphocytes were incubated as described in the legend of Figure 1. Cultures received OKT3 antibody alone or various concentrations of Xerosin alone or a combination of both antibody and drug as indicated. Both dilutions (1/150,000 and 1/250,000) of antibody OKT3 used were sub-optimal. The data in Figure 10 had indicated that Xerosin had an effect only at suboptimal conditions. The results indicate that the effects of antibody OKT3 and Xerosin are synergistic.

APPENDIX II

TABLE 1. Effects of WY 18251 on the proliferation of peripheral blood lymphocytes

OKT3	WY 18251 ($\mu\text{g/ml}$)	[3]Thymidine Incorporation (cpm +/- S.D.)	
-	-	2,058	(385)
+	-	35,684	(8,893)
+	3	29,412	(3,391)
+	1	22,036	(2,101)
+	0.330	18,035	(1,585)

Peripheral blood lymphocytes were incubated at 2×10^5 cells per microtiter tray well. The monoclonal antibody OKT3 was in the form of tissue culture supernatant and it was used at a dilution of 1/200,000. Cultures were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2 -95% air. Proliferation was assessed by the addition of [3]H-thymidine ($1\mu\text{Ci/well}$) during the last 5 hours of the incubation.

TABLE 2. Simultaneous addition of Xerosin and CL246.738 on lymphocyte proliferation

CL246.738 ($\mu\text{g/ml}$)	[3]Thymidine Incorporation (cpm +/- SD)	
	OKT3	OKT3+Xerosin
0	24,378 (3,872)	44,451 (278)
1.00	24,404 (4,089)	45,262 (1,953)
0.33	16,155 (2,136)	42,698 (3,851)
<hr/>		
Negative Control (no additions):	5,647 (572)	
Xerosin Control (cells + Xerosin only):	7,210 (1,872)	

Culture conditions and concentration of antibody OKT3 were as described in Table 1. Xerosin was used at $3\mu\text{g/ml}$.

TABLE 3. Effects of Xerosin on purified T lymphocyte proliferation.

OKT3	IL-2	Xerosin	[3]H-Thymidine Incorporation (cpm +/- S.D.)	
-	-	-	554	(117)
1/8	-	-	682	(85)
1/32	-	-	437	(113)
1/500	-	-	617	(62)
1/1000	-	-	738	(211)
-	+	-	2,215	(212)
-	-	+	534	(163)
1/8	-	+	822	(242)
-	+	+	1855	(229)
1/8	+	-	38,581	(1846)
1/32	+	-	36,938	(2,015)
1/500	+	-	8,394	(1,271)
1/1000	+	-	8,675	(1,123)
1/8	+	+	44,014	(1,740)
1/32	+	+	45,757	(1,970)
1/500	+	+	10,401	(1,073)
1/1000	+	+	9,022	(965)

The purification of T lymphocytes was performed by rosetting with sheep red blood cells and separation through Ficoll-Hypaque density gradients (see original proposal). Antibody OKT3 was used as a hybridoma culture supernatant at the dilutions indicated. Recombinant IL-2 was used at 30 Units/ml and Xerosin at 3 μ g/ml. All culture conditions and measurement of

TABLE 4

Summary of the effects of drugs on lymphocyte proliferation and surface antigen expression

Drug	Proliferation		Surface Antigen				
	Optimal	Suboptimal	CD3	CD2	CD8	CD4	CD25
CL246	0	Up	0	0	0	0	Down
CL259	0	0	0	0	0	0	0
AbuMDP	0	0	0	0	0	0	0
Xerosin	0	Up	0	0	0	0	0
FK 565	Down	Down	0	0	0	0	0
OK 432	Down	Down	0	Down	0	Up	Up
WY 18251	N.T.	Down	0	0	0	Up	0
AVS 2141	Down	0	0	0	0	0	0
AVS 2142	Down	Down	0	0	0	0	0
AVS 2143	Up	0	0	0	0	0	0
AVS 2144	0	0	0	0	0	0	0

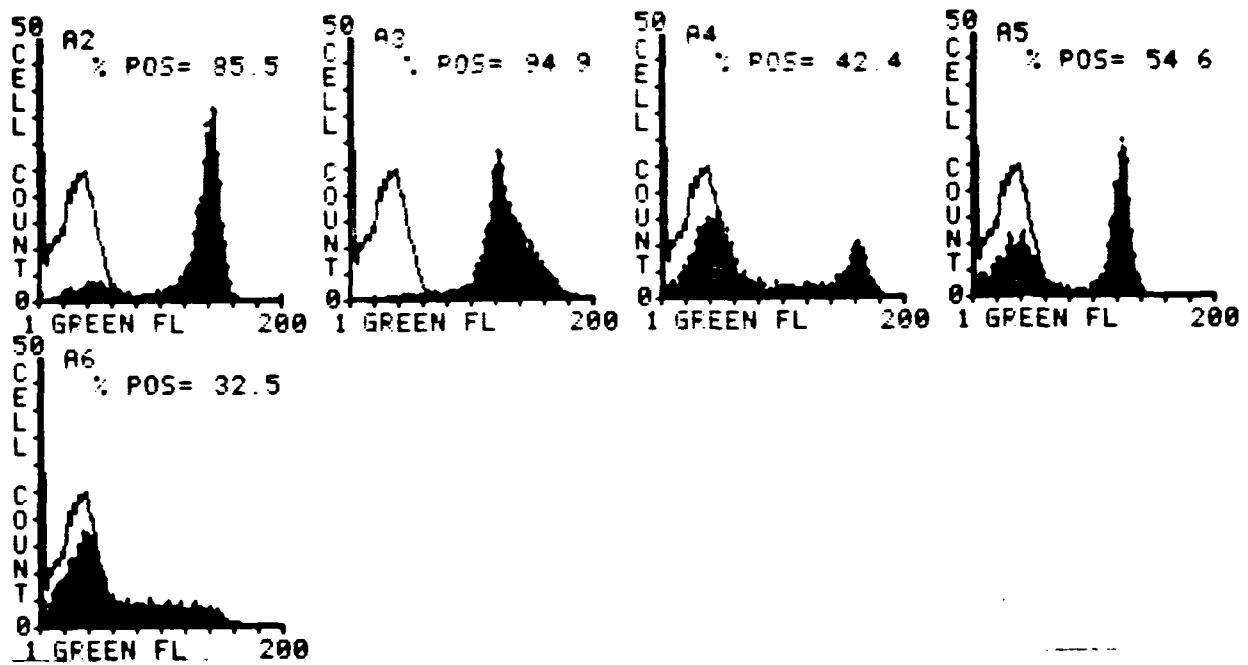
APPENDIX III

LEGEND TO APPENDIX III

The percentages of positive cells are calculated by computer integration of the histogram data.

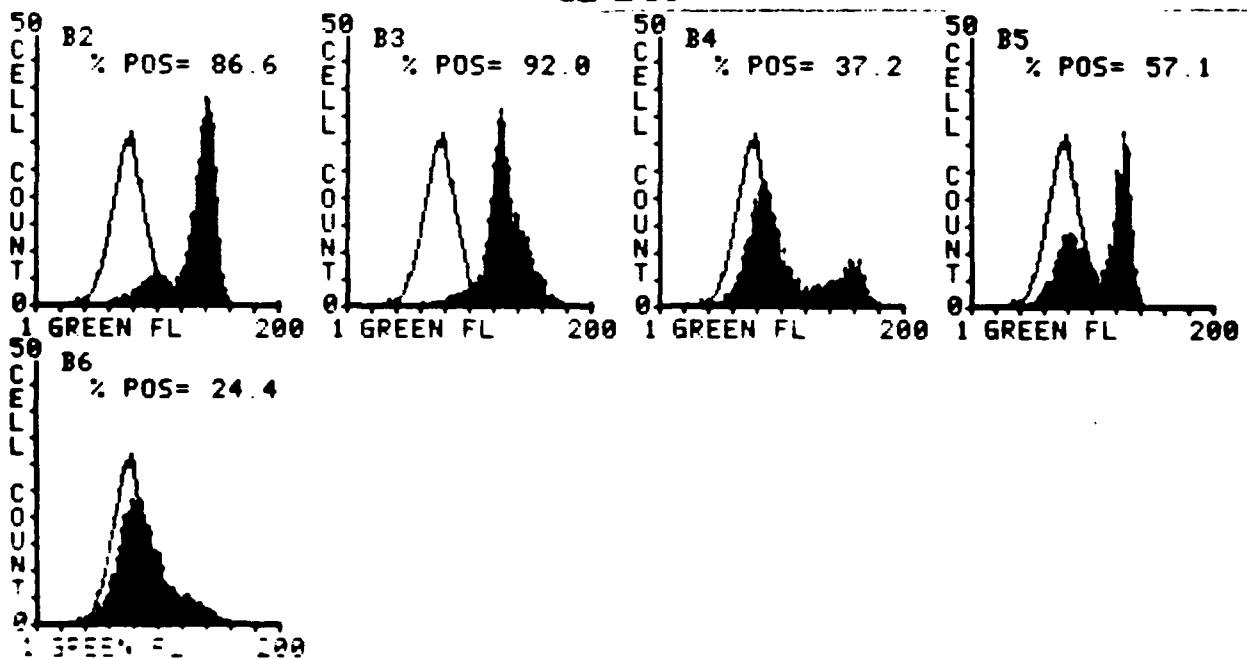
Figure 1A

No drug control



A2=anti-CD3, A3=anti-CD2, A4=anti-CD8, A5=anti-CD4, A6=anti-CD25

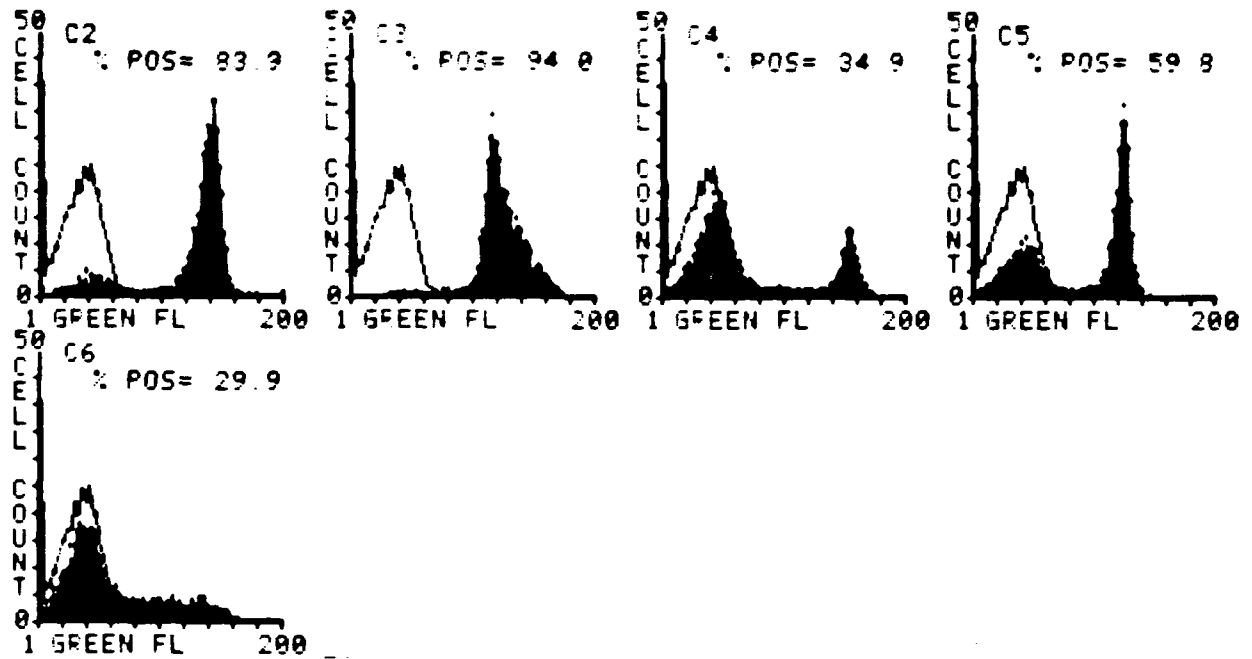
CL 246



B2=anti-CD3, B3=anti-CD2, B4=anti-CD8, B5=anti-CD4, B6=anti-CD25

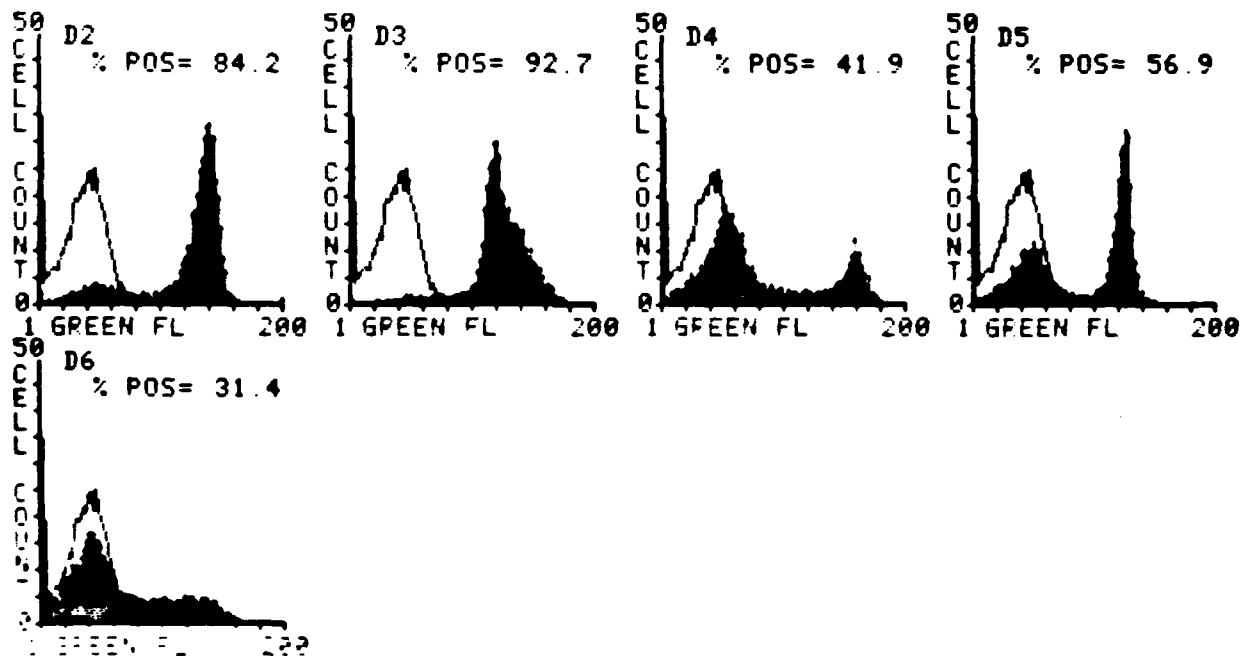
Figure 1B

CL 259



C2=anti-CD3, C3=anti-CD2, C4=anti-CD8, C5=anti-CD4, C6=anti-CD25

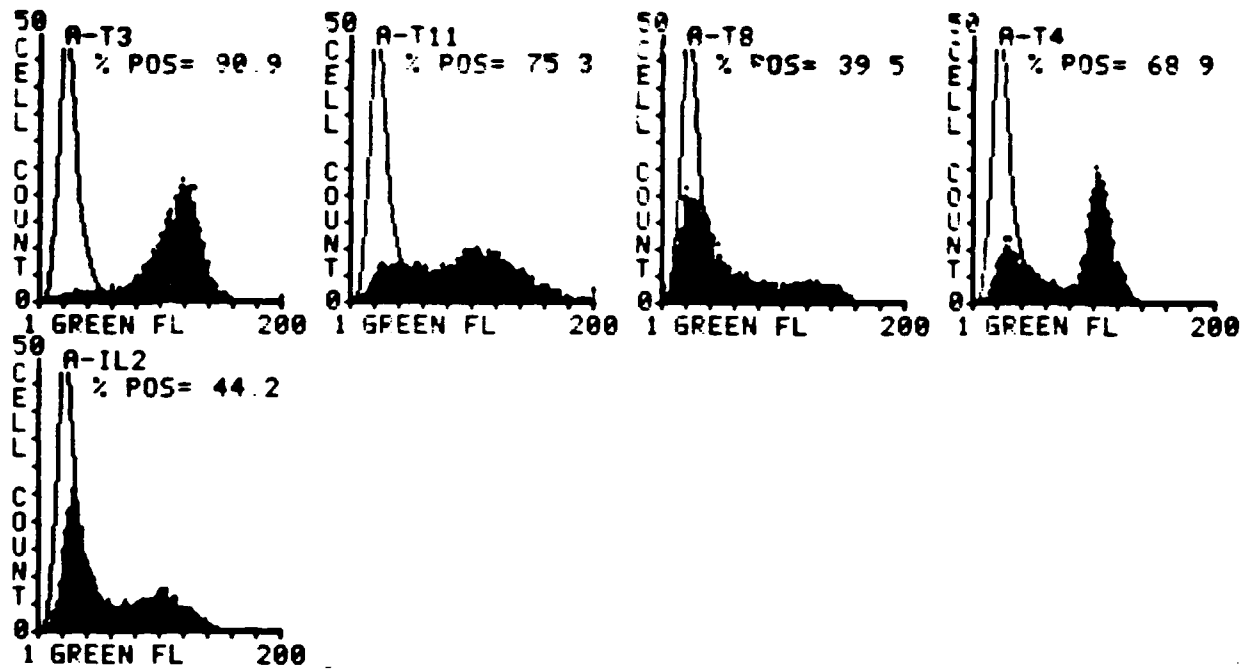
AbuMDP



D2=anti-CD3, D3=anti-CD2, D4=anti-CD8, D5=anti-CD4, D6=anti-CD25

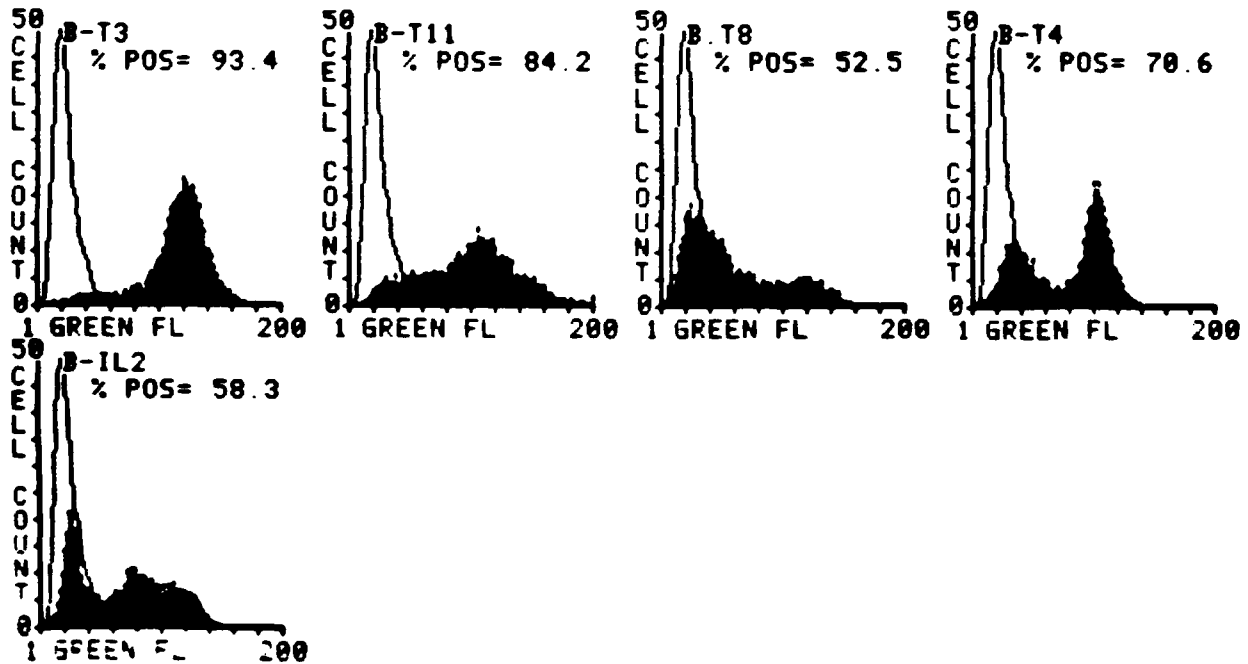
Figure 2A

No drug control



A-T3-anti-CD3, A-T11-anti-CD2, A-T8-anti-CD8, A-T4-anti-CD4, A-IL2-anti-CD25

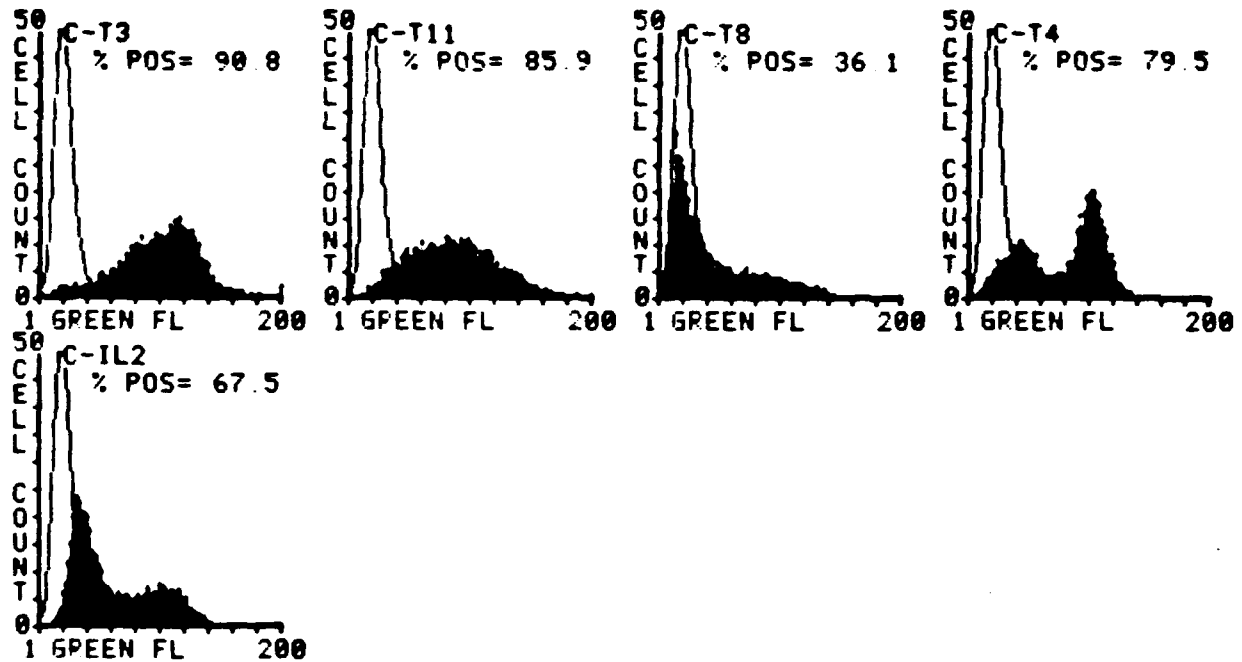
Xerosin



B-T3-anti-CD3, B-T11-anti-CD2, B-T8-anti-CD8, B-T4-anti-CD4, B-IL2-anti-CD25

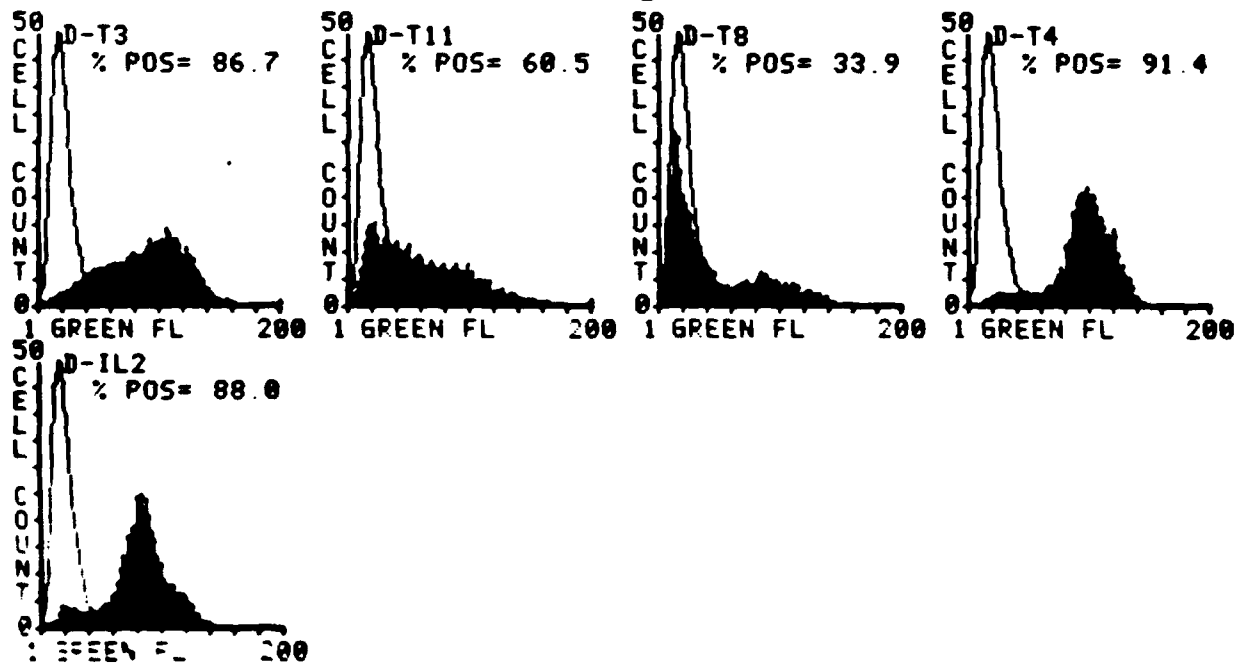
Figure 2B

FK565



C-T3-anti-CD3, C-T11-anti-CD2, C-T8-anti-CD8, C-T4-anti-CD4, C-IL2-anti-CD25

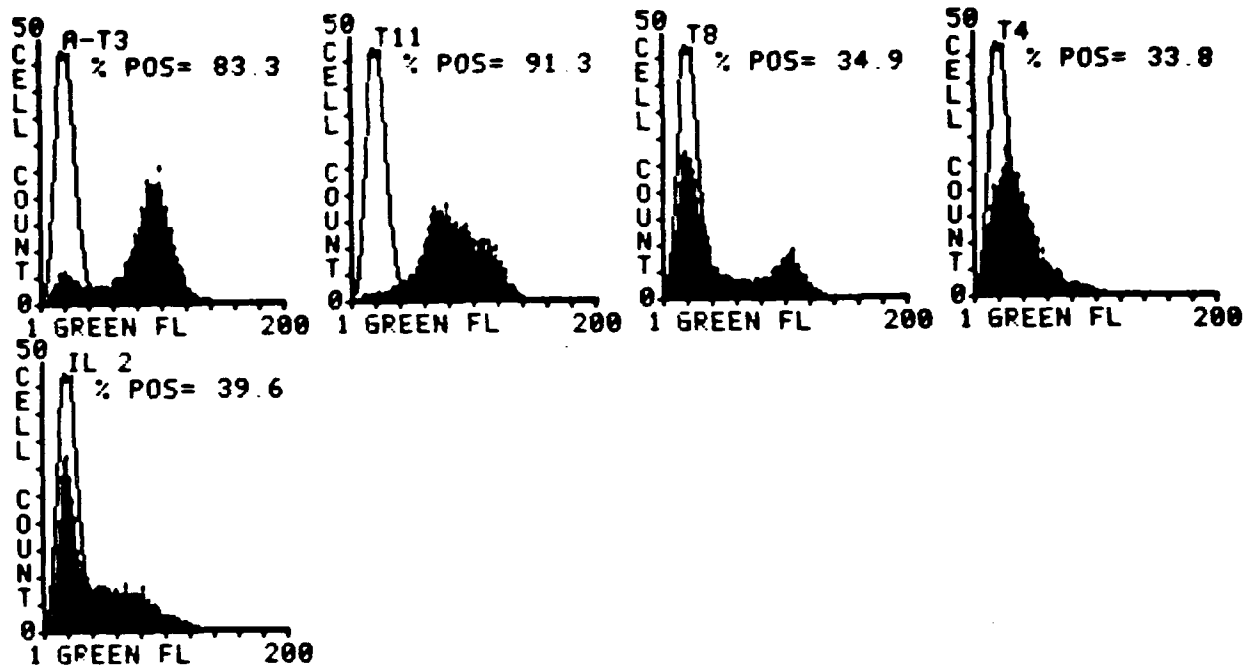
OK432



D-T3-anti-CD3, D-T11-anti-CD2, D-T8-anti-CD8, D-T4-anti-CD4, D-IL2-anti-CD25

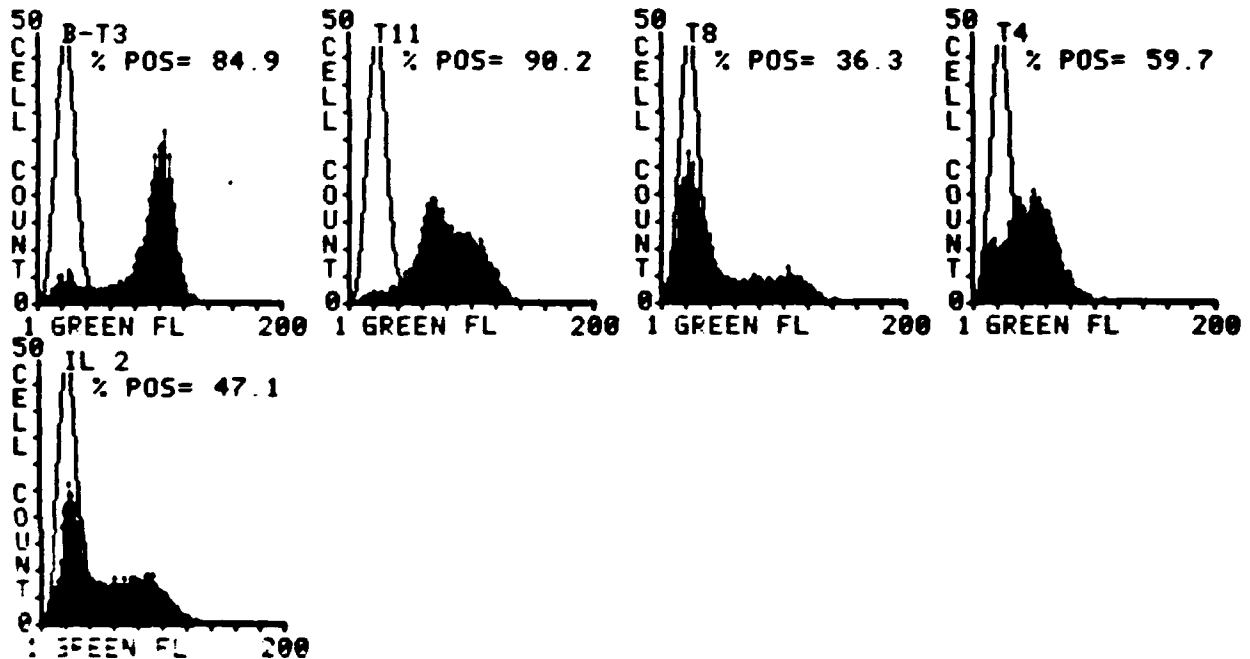
Figure 3A

No drug control



A-T3-anti-CD3, A-T11-anti-CD2, A-T8-anti-CD8, A-T4-anti-CD4, A-IL2-anti-CD25

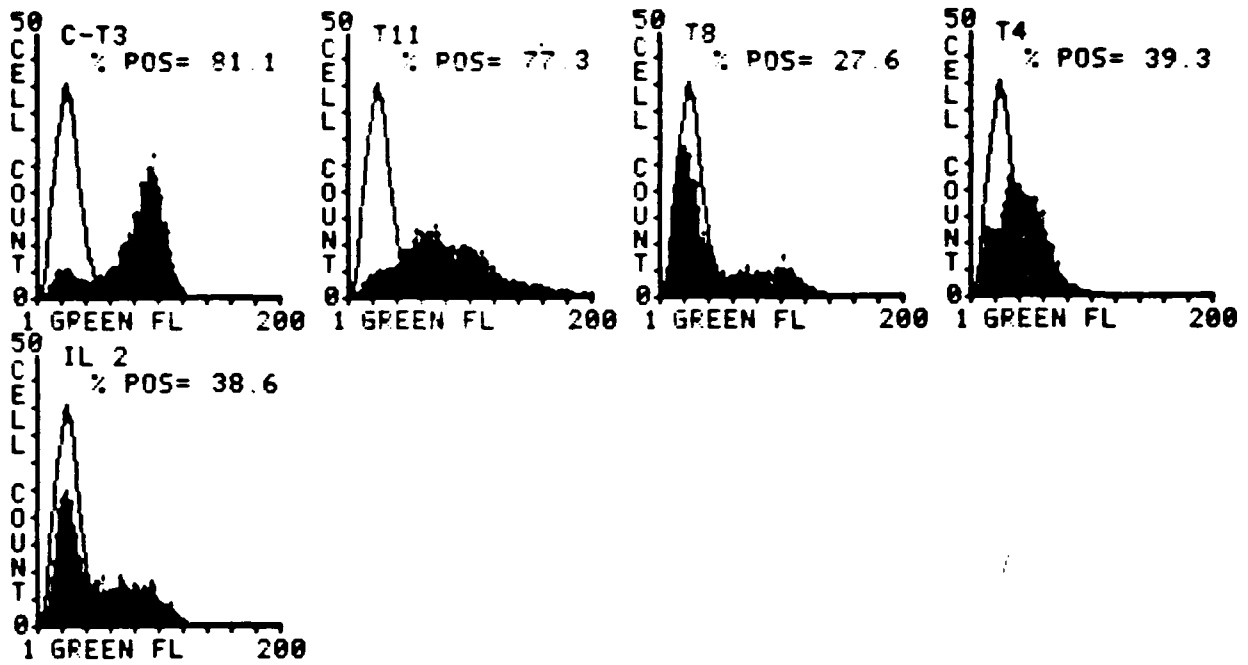
WY18251



B-T3-anti-CD3, B-T11-anti-CD2, B-T8-anti-CD8, B-T4-anti-CD4, B-IL2-anti-CD25

Figure 3B

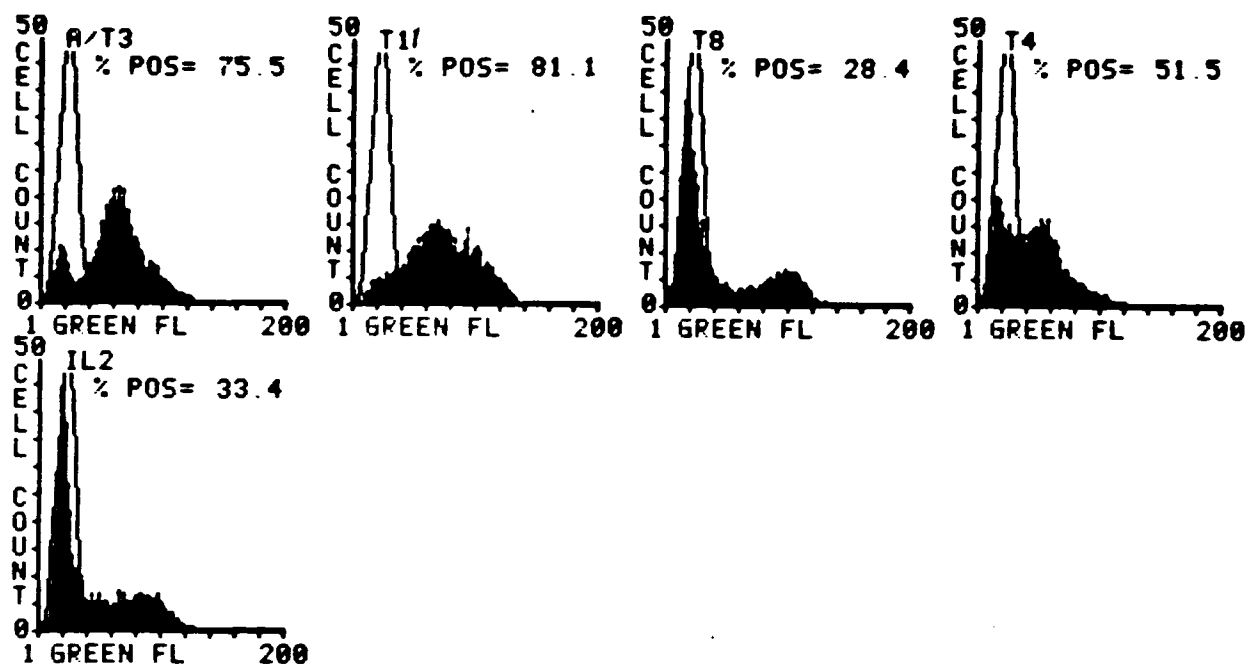
AVS2144



C-T3-anti-CD3, C-T11-anti-CD2, C-T8-anti-CD8, C-T4-anti-CD4, C-IL2-anti-CD25

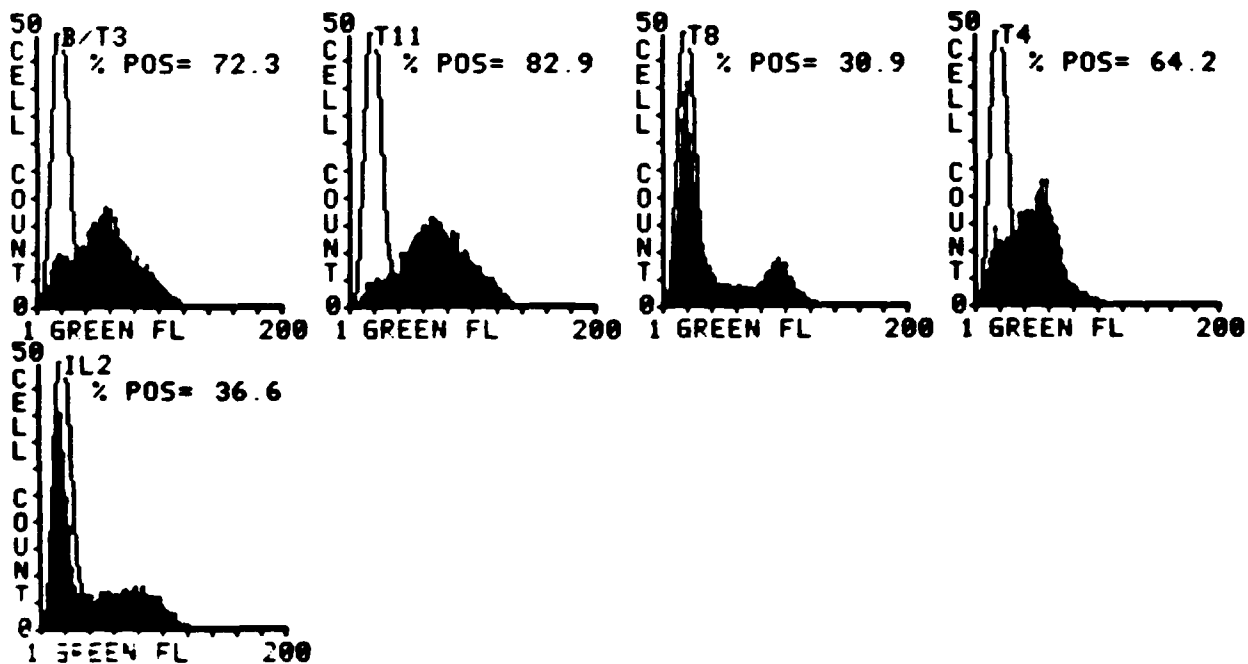
Figure 4A

No drug control



A-T3=anti-CD3, A-T11=anti-CD2, A-T8=anti-CD8, A-T4=anti-CD4, A-IL2=anti-CD25

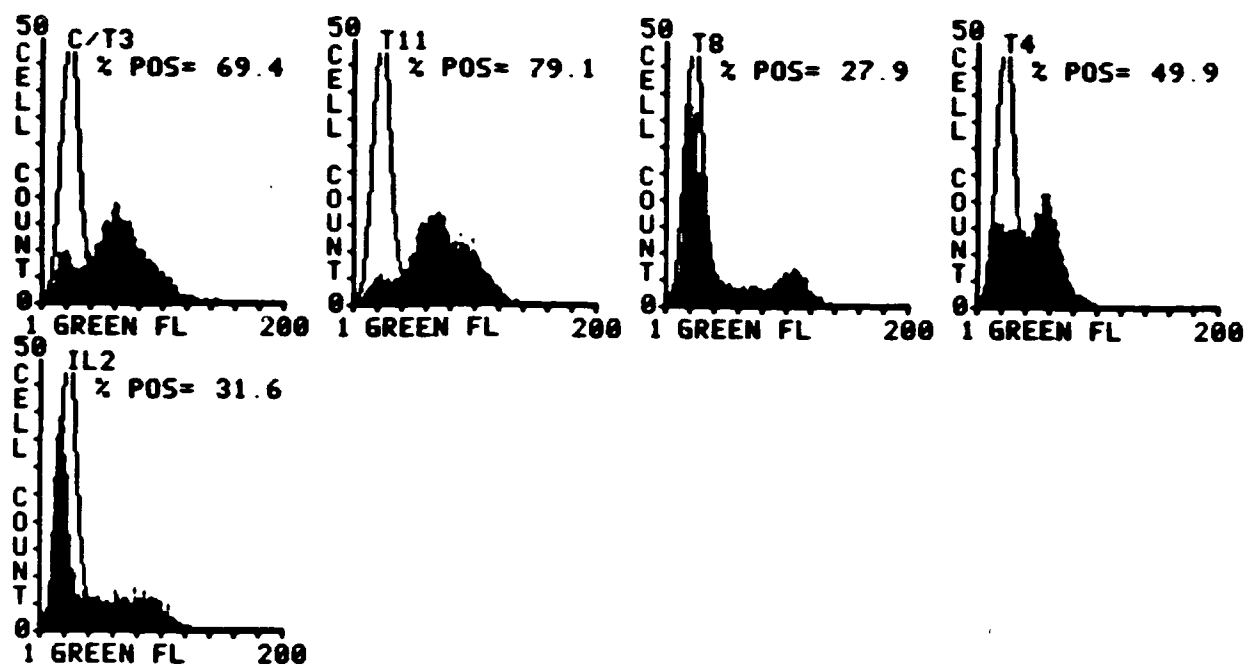
AVS2141



B-T3=anti-CD3, B-T11=anti-CD2, B-T8=anti-CD8, B-T4=anti-CD4, B-IL2=anti-CD25

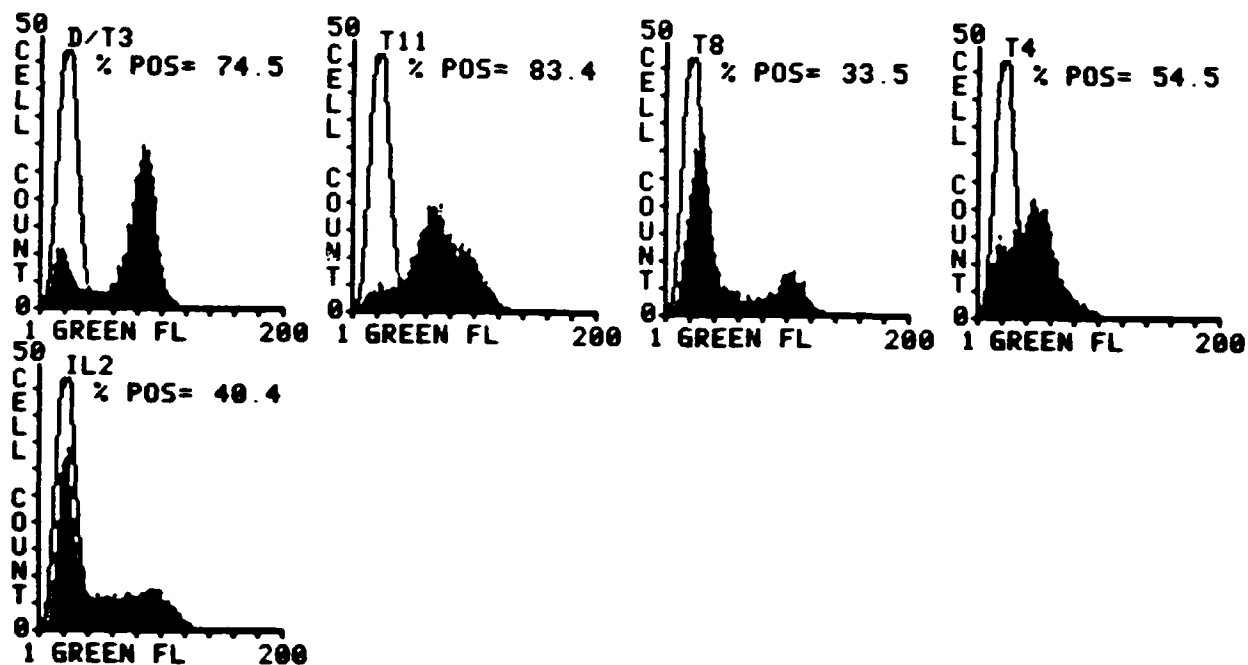
Figure 4B

AVS2142



C-T3-anti-CD3, C-T11-anti-CD2, C-T8-anti-CD8, C-T4-anti-CD4, C-IL2-anti-CD25

AVS2143



D-T3-anti-CD3, D-T11-anti-CD2, D-T8-anti-CD8, D-T4-anti-CD4, D-IL2-anti-CD25

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